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FLUOROCARBON TOXICANT ACTION ON A MEMBRANE CHANNEL  
EFFECTS OF FORNYCIN DE. (U) TENNESSEE UNIV KNOXVILLE  
MEMORIAL RESEARCH CENTER AND HOSPITA. P M WIGLER

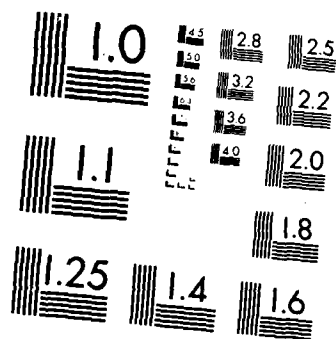
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FINAL TECHNICAL REPORT: AFOSR 82-0261 A through F (four years)  
1 August 1982 to 31 July 1986

**AFOSR-TR- 86 - 0926**

Fluorocarbon Toxicant Action on a Membrane Channel,  
Effects of Formycin Derivatives, Cell Recovery,  
and Detoxification

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Submitted to: Air Force Office of Scientific Research on 16 September 1986

| TABLE OF CONTENTS                               | PAGE  |
|---|-------|
| 1) Summary                                      | 2     |
| 2) Research Objectives                          | 3     |
| 3) Status of the Research                       | 4-15  |
| 3-a Tables                                      | 16-20 |
| 3-b Figures                                     | 21-26 |
| 4) Publications                                 | 27    |
| 5) Professional Personnel                       | 27    |
| 6) Papers Presented at Meetings and Conferences | 27-28 |
| 7) Inventions or Patents                        | 28    |

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SECURITY CLASSIFICATION OF THIS PAGE

## REPORT DOCUMENTATION PAGE

| 1a. REPORT SECURITY CLASSIFICATION<br>UNCLASSIFIED  |   | 1b. RESTRICTIVE MARKINGS   |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
|---|---|--|--------------------------|------------------------|----------------|-------------|------------------|--------|------|----|--|--|--|--|--|
| 2a. SECURITY CLASSIFICATION AUTHORITY   |   | 3. DISTRIBUTION/AVAILABILITY OF REPORT<br>Approved for public release;<br>distribution unlimited.  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 2b. DECLASSIFICATION/DOWNGRADING SCHEDULE   |   |  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 4. PERFORMING ORGANIZATION REPORT NUMBER(S)   |   | 5. MONITORING ORGANIZATION REPORT NUMBER(S)<br><b>AFOSR-TR- 86-0926</b>  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 6a. NAME OF PERFORMING ORGANIZATION<br>University of Tennessee<br>Memorial Research Center  | 6b. OFFICE SYMBOL<br>(If applicable)<br>Medical Biology | 7a. NAME OF MONITORING ORGANIZATION<br>Air Force Office of Scientific Research/NL  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 6c. ADDRESS (City, State and ZIP Code)<br>1924 Alcoa Highway<br>Knoxville, TN 37920   |   | 7b. ADDRESS (City, State and ZIP Code)<br>Building 410<br>Bolling AFB, DC 20332-6448   |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 8a. NAME OF FUNDING/SPONSORING<br>ORGANIZATION<br>AFOSR   | 8b. OFFICE SYMBOL<br>(If applicable)<br>NL              | 9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER<br>AFOSR 82-0261 A-LO-T  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 8c. ADDRESS (City, State and ZIP Code)<br>Building 410<br>Bolling AFB, DC 20332   |   | 10. SOURCE OF FUNDING NOS. <table border="1"><tr><th>PROGRAM<br/>ELEMENT NO.</th><th>PROJECT<br/>NO.</th><th>TASK<br/>NO.</th><th>WORK UNIT<br/>NO.</th></tr><tr><td>61102F</td><td>2312</td><td>A5</td><td></td></tr></table> |                          | PROGRAM<br>ELEMENT NO. | PROJECT<br>NO. | TASK<br>NO. | WORK UNIT<br>NO. | 61102F | 2312 | A5 |  |  |  |  |  |
| PROGRAM<br>ELEMENT NO.  | PROJECT<br>NO.  | TASK<br>NO.  | WORK UNIT<br>NO.         |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 61102F  | 2312  | A5   |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 11. TITLE (Include Security Classification) Effects of Chemicals<br>on the Cell Membrane Transport of Nucleosides   |   |  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 12. PERSONAL AUTHOR(S)<br>Paul W. Wigler, Ph.D., Professor  |   |  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 13a. TYPE OF REPORT<br>Final  | 13b. TIME COVERED<br>FROM 8-1-82 TO 7-31-86             | 14. DATE OF REPORT (Yr., Mo., Day)<br>1986, Sept. 16   | 15. PAGE COUNT<br>28     |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 16. SUPPLEMENTARY NOTATION  |   |  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 17. COSATI CODES <table border="1"><tr><th>FIELD</th><th>GROUP</th><th>SUB. GR.</th></tr><tr><td></td><td></td><td></td></tr><tr><td></td><td></td><td></td></tr><tr><td></td><td></td><td></td></tr></table>   |   | FIELD  | GROUP                    | SUB. GR.               |                |             |                  |        |      |    |  |  |  | 18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)<br>Inhibition of purine efflux by perfluorodecanoic acid, high<br>speed transport assay, uric acid, L5178Y cells, 2-aminopurine,<br>a fluorescent transport substrate, formycin, dilazep |  |
| FIELD   | GROUP   | SUB. GR.   |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
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| 19. ABSTRACT (Continue on reverse if necessary and identify by block number)<br>Treatment of L5178Y mouse lymphoma cells with perfluoro-n-decanoic acid (PFDA) at 30°C produced inactivation of a channel in the cell membrane; activity of the channel was estimated from the efflux of 2-aminopurine (AP). The cells were preloaded with 100 µM AP, put in a flow system, and AP efflux was estimated continuously at 21°C from the fluorescence emission of AP at 370 nm. The initial rate of AP efflux for control cells increased with AP concentration; the reaction was not saturated at 1000 µM AP. The efflux of AP was inhibited by the presence of uric acid in the external buffer with an apparent inhibition constant value of 355 µM for urate. These observations indicate a urate-sensitive channel for AP in the membrane of L5178Y cells. The AP channel was markedly inactivated by 150 µg/ml PFDA for 24 hr at 30°C. There was no significant recovery of AP flux after 3 days at 30°C in fresh growth medium; however, recovery was significant after 6 days. Recovery of activity of the AP channel occurred in one day at 37°C. Cell recovery studies were continued by experiments to show the |   |  |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 20. DISTRIBUTION/AVAILABILITY OF ABSTRACT<br>UNCLASSIFIED/UNLIMITED <input checked="" type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS <input type="checkbox"/>   |   | 21. ABSTRACT SECURITY CLASSIFICATION<br>Unclassified   |                          |                        |                |             |                  |        |      |    |  |  |  |  |  |
| 22a. NAME OF RESPONSIBLE INDIVIDUAL<br>Lt Col Lorris Cockerham  |   | 22b. TELEPHONE NUMBER<br>(Include Area Code)<br>(202)767-5021  | 22c. OFFICE SYMBOL<br>NL |                        |                |             |                  |        |      |    |  |  |  |  |  |

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effect of the drug dilazep on the recovery of L5178Y cells from treatment with three nucleoside toxins. A one-day treatment at 37°C with formycin A, formycin B, or 5'deoxyformycin A produced an arrest in cell growth. The capacity for cell division recovered during two additional days of incubation at 37°C. Cell recovery from each of the three formycin derivatives was accelerated by a simultaneous treatment with dilazep. The cell recovery technique is suggested as a potential procedure to demonstrate toxicant selectivity for different cell types.

Seminar to staff of U.S. Army Medical Research Institute of Chemical Defense, Aberdeen P.G., Maryland, April 18, 1984. "Effect of Perfluorodecanoic Acid on Efflux of 2-Aminopurine from L5178Y Cells".

Seminar to members of the Life Sciences Directorate, AFOSR, Bolling AFB, Washington, DC, April 20, 1984. "Toxicant Effects on Transport of a Fluorescent Purine in L5178Y Cells".

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## 1) Summary:

Treatment of L5178Y mouse lymphoma cells with perfluoro-n-decanoic acid (PFDA) at 30°C produced inactivation of a channel in the cell membrane; activity of the channel was estimated from the efflux of 2-aminopurine (AP). The cells were preloaded with 100  $\mu$ M AP, put in a flow system, and AP efflux was estimated continuously at 21°C from the fluorescence emission of AP at 370 nm. The initial rate of AP efflux for control cells increased with AP concentration; the reaction was not saturated at 1000  $\mu$ M AP. The efflux of AP was inhibited by the presence of uric acid in the external buffer with an apparent inhibition constant value of 355  $\mu$ M for urate. These observations indicate a urate-sensitive channel for AP in the membrane of L5178Y cells. The AP channel was markedly inactivated by 150  $\mu$ g/ml PFDA for 24 hr at 30°C. There was no significant recovery of AP flux after 3 days at 30°C in fresh growth medium; however, recovery was significant after 6 days. Recovery of activity of the AP channel occurred in one day at 37°C. Cell recovery studies were continued by experiments to show the effect of the drug dilazep on the recovery of L5178Y cells from treatment with three nucleoside toxins. A one-day treatment at 37°C with formycin A, formycin B, or 5'deoxyformycin A produced an arrest in cell growth. The capacity for cell division recovered during two additional days of incubation at 37°C. Cell recovery from each of the three formycin derivatives was accelerated by a simultaneous treatment with dilazep. The cell recovery technique is suggested as a potential procedure to demonstrate toxicant selectivity for different cell types.



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2) Research Objectives:

201. To demonstrate that transport channel inactivation is a suitable indicator of in vivo toxicity.

202. To demonstrate that transport channel inactivation is a suitable indicator of in vitro cell toxicity.

203. To show that inactivation of the channel for 2-aminopurine (AP) by perfluorodecanoic acid (PFDA) is a relatively stable lesion in the metabolism of L5178Y mouse lymphoma cells.

204. To demonstrate that cell toxicity is an indicator of in vivo toxicity (see also Olson et al., 1982).

205. To develop a high-speed determination of flux velocity based on fluorescence of a transport substrate.

206. To demonstrate activity of a membrane channel that has not heretofore been observed.

207. To demonstrate recovery of the inactivated transport channel by incubation of the cultured cells in fresh growth medium.

208. To demonstrate channel recovery without cell division.

209. To derive the rate equation for trans inhibition of a membrane channel.

210. To demonstrate trans inhibition by urate of the transport channel for 2-aminopurine.

211a. To develop experimental conditions for the cell recovery technique for toxicant cell-type selectivity.

211b. To demonstrate cell arrest with nucleoside toxins.

211c. To demonstrate the effects of a drug on recovery of cells from effects of nucleoside toxins.

### 3) Status of the Research:

#### 301. Channel Inactivation Indicates In Vivo Toxicity.

One of the most important goals of this project was to support the concept that inactivation of membrane transport is an appropriate "biomarker" for toxicant effects on living animals. To accomplish this goal, it was important to correlate data at the cellular level with the results of whole-animal toxicity studies. Olson and Andersen (1983) showed that PFDA is more toxic to rodents than PFOA. The studies reported by Wigler and Shah (1986) showed a 97.3 percent inactivation of the AP channel by 200  $\mu\text{g/ml}$  PFDA and an 8.5 percent inactivation of the channel by an equivalent concentration of PFOA. Thus, PFDA is considerably more potent in channel inactivation than PFOA. The agreement between the results from whole-animal studies and the studies of membrane transport inactivation support the idea that membrane transport is a good biomarker of in vivo toxicity.

#### 302. Channel Inactivation Indicates In Vitro Cytotoxicity.

The PFDA inactivation of the AP channel may be correlated with the cytotoxicity of PFDA to L5178Y cells. For the acronycine effects on L5178Y cells, Dunn *et al.* (1973) suggested the following criteria for a flux inhibition-cytotoxicity correlation: "...population growth is suppressed...only at acronycine concentrations that markedly inhibit nucleoside utilization; cells returned to drug-free medium...rapidly regain both their ability to take up nucleosides and their normal rate of growth." The data of Table 1 (Wigler and Shah, 1986) provide an opportunity to correlate flux inhibition with the cytotoxic effects of PFDA.

The data of Table 1 show that L5178Y cells grow slowly at 30°C; the cell count for untreated controls increases 8 percent after 24 hr. When the cells are treated with 150  $\mu\text{g/ml}$  PFDA, cell growth is arrested for 24 hr at 30°C. These cells appear to be fully viable, based on the trypan blue exclusion method. Furthermore, after a transfer to fresh growth medium, the PFDA-treated cells grow at approximately the same rate as the controls (see Table 3). It is interesting to note that the capacity for cell division seems to recover faster at 30°C than the capacity for AP flux.

When the cells are treated with 200  $\mu\text{g/ml}$  PFDA in growth medium at 30°C, the cell counts decrease by 12.6 percent after 24 hr. The capacity for AP efflux is markedly decreased with the foregoing experimental treatment. When the cells are grown at 37°C, the cell count of untreated controls increases 44



percent after 24 hr (see Table 4). The report by Rogers *et al.* (1982) indicates that 100  $\mu\text{g/ml}$  PFDA at 37°C produces an arrest in cell division during a 24 hr incubation period. (The increased count of control cells in 24 hr is almost the same as the inhibition of PFDA-treated cells in comparison to controls.) The cytotoxic population growth effect of 100  $\mu\text{g/ml}$  PFDA at 37°C is approximately equal to the effect of 150  $\mu\text{g/ml}$  PFDA at 30°C (see Table 1). Recovery of the PFDA-inactivated AP channel occurs slowly at 21°C. Since there is no significant cell division at this temperature, the AP channel of parent L5178Y cells is capable of recovery.

A preliminary analysis of the L5178Y cell population was performed with a high efficiency flow cytometer. A small population of enlarged cells was observed after a 24 hr incubation of control cells at 30°C; the enlarged cells were eliminated by treatment with PFDA. Furthermore, a small population of tetraploid cells was observed after a 24 hr incubation of control cells. The number of tetraploid cells increased as the concentration of PFDA was increased. These observations suggest that the  $G_2$  stage of the cycle of cell division is blocked by PFDA.

The concentrations of PFDA to produce a cytotoxic effect on cell division are of the same magnitude as the concentrations required to inactivate the AP channel. This observation suggests a potential correlation between transporter inactivation and cytotoxicity. An inhibition of the excretion of the toxic end products of catabolism, such a uric acid, could produce a delayed cytotoxicity.

The results of Table 1 indicate that a treatment with 200  $\mu\text{g/ml}$  perfluorooctanoic acid (PFOA) for 24 hr at 30°C has no significant effect on the cell count or AP efflux in comparison with the controls. By contrast, treatment with 200  $\mu\text{g/ml}$  PFDA produces a marked inactivation of the AP channel and a drop in cell count from  $19.0 \times 10^5$  to  $16.6 \times 10^5$  cells/ml. Thus, it is apparent that changes in channel activity are reflected in corresponding changes in cytotoxicity.

When the PFDA studies on L5178Y cells were initiated in this laboratory (1983), we tried to use the same experimental conditions as the studies performed at Wright-Patterson AFB (Rogers *et al.*, 1982). The group in Ohio used DMSO to solubilize the PFDA or PFOA in the growth medium for L5178Y cells and an incubation temperature of 37°C. Because of the potential effects of DMSO on membrane permeability, we used ethanol as the solubilizer for PFDA. It is

difficult to predict whether DMSO may influence the cytotoxicity of the fluorocarboxylic acids.

At the beginning of this project, an experiment was performed to determine the effect of prior incubation with 100  $\mu\text{g/ml}$  PFDA in medium plus serum for 24 hr at 37°C. As shown in Fig. 1, efflux of AP by PFDA-treated cells is slower than efflux by control cells. It was of interest to compare the effects of prior incubation of L5178Y cells with PFDA at 37°C with the results of a similar experiment at 30°C. When a concentration of 100  $\mu\text{g/ml}$  PFDA is used for 24 hr at 30°C, the effect on AP efflux is about the same as at 37°C (see Table 1, Wigler and Shah, 1986). In the experiments performed in this laboratory, however, it was impossible to investigate transport with PFDA concentrations above 100  $\mu\text{g/ml}$  at 37°C because most of the cells were lysed. Thus, a total inactivation of AP carrier activity could not be estimated at 37°C. Inactivation of AP efflux could be reproduced with a range of PFDA concentrations at 30°C, however, with minimal morphological effect on the L5178Y cells.

#### 303. Channel Inactivation by PFDA Is a Stable Lesion to L5178Y Cells.

At the end of a 1 day treatment with 150  $\mu\text{g/ml}$  PFDA, the AP efflux activity is 19.5% of controls (Table 1) and, at the end of three additional days at 30°C, the activity is 22.5% of controls (Table 3; Wigler and Shah, 1986). Thus, the inactivation of the AP channel persists for 3 days after the PFDA is removed from the growth medium. During the third day of recovery, the PFDA-treated cells were growing faster than the controls. This was a consistent finding; after a lag in cell growth, the PFDA-treated cells would grow faster than the controls (see Table 4).

#### 304. Cytotoxicity Indicates In Vivo Toxicity.

The studies of Olson et al. (1982) showed that PFDA is more toxic to rats than PFOA. In further studies on the growth of L5178Y cells at 37°C, these authors showed that PFDA is more cytotoxic than PFOA. At a dose of 100  $\mu\text{g/ml}$  PFDA, the counts of L5178Y cells in suspension are 53% of the control; at the same concentration of PFOA, the counts are 76% of controls (Olson et al., 1982). The same experiment was performed with 200  $\mu\text{g/ml}$  PFDA or PFOA at 30°C by Wigler and Shah (1986). The data of Table 1 show that at 200  $\mu\text{g/ml}$  PFDA, the counts of L5178Y cells are 81.0% of controls and at the same concentration of PFOA (30°C), the counts are 95.6% of controls. (It is important to note that it was not possible to perform the AP efflux studies at 37°C.

See section 302.) The studies performed in this laboratory provide confirmation of the results of Olson et al. (1982).

305. To Develop a High-Speed Determination of Flux Velocity Based on Fluorescence.

Experiments were conducted in 1982 to determine the initial rate of AP efflux from L5178Y cells, using an aliquot and quench assay procedure. At best, individual time points could be taken at 8 sec intervals and the high pipetting error interfered with the velocity determination.

Determinations of the initial velocity of membrane transport require a high speed kinetic procedure. A prototype filtration-flow apparatus was constructed by the Principal Investigator from scrap metal and routine laboratory parts; a sketch of the apparatus is shown in Fig. 2. An assembly that consists of a hypodermic barrel and a membrane filter holder was mounted on top of the cell compartment lid of the fluorescence spectrophotometer with a tripod. The filter effluent drains into a quartz fluorescence flow cell through medical plastic tubing. The outflow plastic tubing from the quartz cell passes through a pump to regulate the rate of flow. Excitation light illuminates the flow cell from behind, and the emission light is detected at a rectangle with a high sensitivity photomultiplier tube. It was possible to measure the emission light at 2 sec intervals and the SLM instrument is able to store the kinetic data at that speed.

There is an inherent advantage to flux measurements from a small volume into a large volume in the direction of efflux. Furthermore, an assay based on fluorescence can detect substrate molecules at very low concentrations. Thus, the assay for membrane transport described in this report is one of the best methods of estimation of substrate flux ever developed.

L5178Y cells preloaded with AP were mixed with a solution of uric acid (at a final concentration of 500  $\mu$ M) in PBS buffer at 21°C. The resultant suspension was quickly poured into the flow-filtration apparatus and the effluent buffer was excited with light at 312.5 nm (a wavelength of high emission for the mercury lamp). The emission data was corrected for a cell count of  $10^{12}$  cells per liter, based on counts of each cell suspension. The rate of increase in emission at 370 nm (the peak for AP) was reported (Wigler and Shah, 1986). The experiment was repeated with the addition of 500  $\mu$ M of hypoxanthine, adenine, xanthine, or no addition (control). Although the efflux of AP is inhibited by uric acid in the external buffer, the addition of

adenine, hypoxanthine, or xanthine at 500  $\mu$ M has no significant effect on the rate of AP efflux in comparison with the control. Guanine and isoguanine could not be tested with this system because these compounds are insoluble at 500  $\mu$ M concentration in aqueous buffer at pH 7.4.

In a separate experiment, PFDA was tested as a potential trans (direct) inhibitor of AP efflux. The preloaded cell pellet was resuspended in a PFDA solution in PBS at 21° and the suspension was poured into the flow-filtration apparatus. The results indicate that PFDA at 100  $\mu$ g/ml or 200  $\mu$ g/ml in the external buffer has no direct effect on AP efflux in comparison with the controls. In this experiment, excitation of the effluent was at 308 nm, the peak excitation wavelength for AP. (The emission from a standard solution of AP at the Hg emission of 312.5 nm excitation was elevated 25% in comparison with excitation at 308 nm.)

#### 306. To Demonstrate Activity of a Novel Membrane Channel.

The method for determination of purine flux described in section 305 is a new procedure. The uptake of high concentrations of 2-aminopurine (AP) was reported by Caras et al. (1982). At the high substrate concentrations employed, it was not possible to distinguish between flux by diffusion through the plasma membrane lipids, a facilitated diffusion process, or flux through a membrane channel. The studies of Wigler and Shah (1986) clearly show that the flux of AP occurs through a membrane channel. This is the first experimental demonstration of the AP membrane channel.

#### 307. Channel Recovery in Fresh Growth Medium.

The PFDA-treated L5178Y cells were incubated in fresh medium for periods up to seven days and the cells were tested for recovery of the AP channel. Very little channel recovery was observed after 3 days at 30°C; subsequently, the rate of AP efflux increased. The rate of channel recovery was investigated at three different temperatures. We observed that the L5178Y cells could be incubated at room temperature (21°C) in growth medium for one week with no apparent damage to the cells. The channel recovers slowly at 21°C and 30°C, but at 37°C, channel recovery can be demonstrated in one day (see Table 4).

#### 308. Channel Recovery Does Not Require Cell Division.

L5178Y cells are rather unusual because they can be incubated at relatively low temperatures without a loss in cell viability. The cells were incubated at room temperature (21°C) for seven days; tests for viability by

the trypan blue exclusion method showed that these cells are fully viable. At 21°C, there is virtually no division of L5178Y cells. These cell characteristics provided an opportunity to determine whether the AP channel of parent cells can recover. (At 30°C and 37°C, the recovery of cell growth and the recovery of the AP channel activity occur together.) As shown in Table 4 of Wigler and Shah (1986), the AP channel recovers at 21°C when there is virtually no cell division.

### 309. To Derive a Rate Equation for Trans Inhibition of a Channel.

The rate equation reported in Wigler and Shah (1986) is original and probably the only mechanism study of trans inhibition of a channel so far reported. There were several problems associated with understanding the trans inhibition mechanism of a channel. Our experiments with different substrate concentrations showed clearly that the flux of AP is a nonsaturable phenomenon up to 1000  $\mu$ M AP. Thus, there appears to be no binding site for AP inside the channel. The nonsaturability of the substrate also eliminates the possibility of a gated-carrier mechanism in AP flux. The postulated Michaelis-Menten channel-substrate complex indicates that the substrate (AP) is inside the channel, but not bound to a receptor. Our experiments with uric acid indicated that the plot of  $1/v$  against  $[I]$  gives two parallel straight lines at two different substrate concentrations. (The symbol  $[I]$  represents the concentration of the inhibitor, uric acid.) To satisfy the need for parallelism, it was necessary to suggest that the inhibitor can dissociate from the channel only when the substrate is inside the channel. Another way of looking at this concept is that the inhibitor only influences the rate of substrate flux when the substrate is inside the channel and the inhibitor is bound to a receptor inside the channel. The postulated urate-channel-substrate complex gives two parallel lines in the  $1/v$  against  $[I]$  plot. The rate equation of trans inhibition of the AP channel is consistent with the experimental results for urate inhibition (see section 310).

### 310. Trans Inhibition by Urate of the AP Channel.

A trans inhibition experiment was performed with L5178Y cells preloaded with AP and suspended in solutions of uric acid in FBS buffer at 21°C. The resultant suspension were quickly poured into the flow-filtration apparatus and the effluent buffer was excited with light at 312.5 nm (a wavelength of high emission for the mercury lamp). The emission data was corrected for a cell count of  $10^{12}$  cells per liter, based on counts of each cell suspension.

The effect of time on the increase in emission at 370 nm (the peak for AP was determined). The efflux of AP at 300  $\mu$ M and 600  $\mu$ M AP was determined with five different concentrations of uric acid in the external buffer (from 200  $\mu$ M to 800  $\mu$ M). The uric acid concentration is plotted against the reciprocal of the initial efflux velocity of AP in Fig. 3. Inspection of the figure indicates two parallel straight lines (see equation 4). The data from the uric acid experiment were evaluated by an analysis of least squares and a test for parallel lines.

311a. To Develop Experimental Conditions for the Cell Recovery Technique for Toxicant Cell-Type Selectivity.

311b. To Demonstrate Cell Arrest with Nucleoside Toxins.

311c. To Demonstrate the Effects of a Drug on Recovery of Cells from Effects of Nucleoside Toxins.

Cultured animal cells that have been partially damaged by a toxicant can recover if the cells are transferred to fresh growth medium (Wigler and Shah, 1986). Further information on the mechanisms of cell recovery could provide useful therapeutic information. For example, it may be possible to enhance the recovery process by the use of pharmaceutical agents and/or hormones. Earlier studies from this laboratory show that the recovery of PFDA-treated L5178Y cells is temperature dependent. Recovery of the capacity for cell division and recovery of the AP channel activity is enhanced by elevated temperatures up to 37°C. The current experiments on cellular recovery have utilized three derivatives of formycin. The structure of the formycin derivatives is shown in Fig. 4.

There are several reasons for the choice of formycins as the toxicants in these experiments. First, the fluorescence of the compound will allow detection of formycin A at low concentrations with the equipment available in this laboratory. Second, the carbon to carbon glycosyl bond in formycins provides a metabolically stable substrate. The formycin A can be incorporated into cellular RNA, DNA, and coenzymes as an analog of adenosine. Turnover of the nucleotide releases free formycin A into the cytoplasm of the cell. Third, the influx and efflux of formycin A is probably mediated by the adenosine carrier system. It may be possible to demonstrate the formycin flux reaction by the fluorescence technique. Fourth, the metabolism of formycin A has been established; the nucleoside is converted to a 5'-phosphate by adenosine kinase and to the 5'-triphosphate by AMP and ADP kinases. The formycin

5'-triphosphate is then incorporated into RNA by a synthetase enzyme. The catabolism of formycin A probably includes deamination by adenosine deaminase to formycin B.

Samples of  $1.0 \times 10^6$  L5178Y cells per ml were incubated at 37°C in growth medium that contained different concentrations of formycin A (from 0.1  $\mu$ M to 10.0  $\mu$ M). At the end of 24 hr and 48 hr incubation periods, the cells were counted and the uptake of trypan blue was determined; the results were compared with controls. The effect of formycin A on the counts of L5178Y cells is shown in Fig. 5.

During a 24 hr incubation of control cells at 37°C, the count increased from  $1.0 \times 10^6$  cells/ml to  $1.6 \times 10^6$  cells/ml. When the cells were treated with increased concentrations of formycin, the cell counts observed after 24 hr were decreased. For a formycin A concentration of 2.0  $\mu$ M, the yield of cells was below the count at time zero, and at 10.0  $\mu$ M formycin A, the cell yield was 15% below the cell count at time zero. The cell count was higher at each formycin level after a 48 hr incubation in comparison with the data for 24 hr. This observation suggests that most of the cell damage by formycin A occurs within 24 hr; subsequently, the cells begin a partial recovery.

Cell viability was determined by the trypan blue method after each incubation with formycin A. The control cells (after 24 hr incubation at 37°C) showed a viability of 95%, and after incubation in 10.0  $\mu$ M formycin the cell viability was 96% (N = 2). The corresponding data for the 48 hr incubation was 90% viability for controls and 94% viability for the cells treated with 10.0  $\mu$ M formycin A. Although formycin A produces an arrest of cell division at concentrations below 10.0  $\mu$ M (see Fig. 5), the formycin has no significant effect on cell viability under these experiment conditions.

Another experiment was performed to determine whether L5178Y cells can recover from a treatment with 50  $\mu$ M formycin A. In this experiment, the cells were treated with 50  $\mu$ M formycin A in growth medium for 2.0 hr at 30°C. It was anticipated that a two-hour incubation is sufficient for the complete uptake of the formycin A into L5178Y cells. In the recovery experiment, cell counts were determined for a period of 7 days.

A sample of  $4.0 \times 10^6$  L5178Y cells in 10 ml was mixed with a formycin A stock solution to give a final concentration of 50  $\mu$ M formycin A. The cell suspension was incubated for 2.0 hr at 30°C with gentle rocking. The suspension was centrifuged at 1000 RPM and the supernatant was decanted. The cell

pellet was resuspended in growth medium twice and centrifuged twice to remove extracellular formycin A. The pellet was resuspended in 10 ml growth medium and incubated at two different temperatures for 7 days. (Fresh medium was added every 3 days to provide the nutrients needed for cell division.)

The formycin-treated cells were rinsed and transferred to fresh medium. The growth of the control cells was delayed for one day after the transfer; subsequently, cell division was rapid at 30°C and 37°C. The formycin-treated cells were arrested at a count of approximately  $0.2 \times 10^6$  cells/ml for more than 2 days at 30°C and 37°C. At 30°C for 7 days, the cell count increased to  $0.6 \times 10^6$  cells/ml; and, at 37°C for 7 days, the count increased to  $1.6 \times 10^6$  cells/ml. Thus, the L5178Y cells can recover from a treatment with 50  $\mu$ M formycin A.

There are two potential mechanisms for the formycin A detoxification and cell recovery. One hypothesis suggests that formycin A is deaminated by adenosine deaminase to formycin B. The deamination reaction could be relatively slow at 30°C; this may be consistent with the lengthy period of cell arrest at this temperature. To investigate this possibility, we have compared the cytotoxicity of formycin A with formycin B. Results indicated that formycin A is a more potent inhibitor of cell division than formycin B. Thus, the deamination of formycin A appears to be a possible mechanism for detoxification and cell recovery. Another hypothesis suggests that detoxification occurs when formycin A or formycin B is excreted from the cell into the medium.

An experiment was performed to compare the effects of formycin A with two formycin derivatives. The L5178Y cells ( $0.5 \times 10^6$  cells/ml) were incubated with formycin A, formycin B, or 5'-deoxyformycin A for two days at 37°C. The final concentration of each inhibitor was 20  $\mu$ M to 160  $\mu$ M and the cell counts were determined at the end of the first and second day; the results are shown in Fig. 6. After one day, the cell count was reduced approximately 50% (in comparison to the controls) by 40  $\mu$ M formycin. The cell count was reduced approximately 50% at the end of day one by 120  $\mu$ M formycin B or 5'-deoxyformycin (gift of Dr. L.B. Townsend). Thus, a 3-fold higher concentration of one of the formycin derivatives is required to produce an arrest in cell division in comparison to formycin A. At formycin concentrations above 40  $\mu$ M, many of the L5178Y cells are destroyed.



At the end of day 2, all of the cell suspensions treated with formycin A show an increase in the cell count. Although these results indicate the recovery of cells treated with formycin A concentrations up to 50  $\mu$ M, the recovery from concentrations of formycin A higher than 50  $\mu$ M may not be significant. On the other hand, the cells treated with 5'-deoxyformycin A show a dramatic recovery in the second day; the cell counts were almost the same as the controls. For the cells arrested with 120  $\mu$ M 5'-deoxyformycin A, this indicates an increase from  $0.5 \times 10^6$  cells/ml at the end of day one to a cell count of  $1.2 \times 10^6$  cells/ml at the end of day 2. Since the 5'-deoxyformycin A cannot be converted to a 5'-nucleotide, it seems probable that cellular excretion is a major mechanism in the cell recovery observed in Fig. 6.

A concentration of 120  $\mu$ M formycin B is required for a complete arrest of cell division for 1 day. During the second day, the cell count doubles, indicating a significant cell recovery process. Since formycin B does not contain an amino group, cellular excretion of the formycin B is the probable mechanism of L5178Y cell recovery.

The effect of the drug dilazep on the recovery of toxin-treated L5178Y cells was demonstrated in a subsequent experiment. The concentration of each formycin derivative was selected to provide cell arrest for approximately one day and a small decrease in cell viability. A treatment with 50  $\mu$ M dilazep alone had no effect on cell viability in comparison with the control; the dilazep treatment produced a moderate inhibition of cell division (see Table 5).

A one-day treatment with 5.0  $\mu$ M formycin A in growth medium at pH 7.4 and 37°C produced a 25% decrease in the cell count; 13% of the cells were nonviable. The combination treatment of formycin A and dilazep produced cell arrest for one day and a significant enhancement of the rate of cell recovery compared with formycin A alone. Based on the concentrations required for cell arrest, the cytotoxicity of formycin B is much lower than that of formycin A. This finding suggests that the deamination of formycin A to formycin B by adenosine deaminase is a probable pathway in detoxification of formycin A. A combination treatment of formycin B plus dilazep produces an enhancement of the rate of cell recovery compared with formycin B alone.

The effects of 5'-deoxyformycin A on the growth and recovery of L5178Y cells are interesting because the effects of this new compound on cells have never been studied. The compound was prepared by Dr. L. Townsend for use in

this project. Although the 5'-deoxy compound is not converted to a 5'-nucleotide by enzymic phosphorylation, the nucleoside has a cytotoxic effect on L5178Y cells. The addition of dilazep two hr after the start of the incubation produces an enhancement in the rate of cell recovery (see Table 5).

Dilazep, a highly water soluble derivative of homopiperazine, blocks the uptake of adenosine into L5178Y cells (Paterson et al., 1984). Paterson et al. (1979) have studied the cell growth effects of another transport inhibitor in combination with nucleoside toxins. In the 1979 study, RPMi 6410 cells were protected from the cytotoxicity of nebularine (9- $\beta$ -D-ribofuranosylpurine) by the transport inhibitor nitrobenzylthioinosine (NBMPR). In these experiments, the NBMPR was added to the cell culture before or simultaneously with the nebularine. If the NBMPR was added one hr after the nebularine, the transport inhibitor was ineffective in protecting the cells from the nucleoside toxin.

As can be seen from Table 5, the results in this laboratory are different from those of Paterson et al. (1979). Dilazep is effective in protecting against the formycin derivatives when it is added two hr after the toxin. The probably explanation of this observation is the metabolic stability of formycin (to nucleoside phosphorylase) in comparison to nebularine. Thus, the formycin A may be excreted from L5178Y cells intact and subsequently taken up again by the cells. The dilazep may inhibit the secondary uptake of the formycin A.

SUMMARY: EFFECTS OF FORMYCINS AND DILAZEP ON GROWTH ARREST AND RECOVERY OF L5178Y CELLS

The effects of nucleoside toxins and the drug dilazep on cell growth, cell viability, and the recovery of cell growth were determined; L5178Y mouse lymphoma cells were treated with a formycin derivative during an incubation at 37°C. Concentrations of 5.0  $\mu$ M formycin A, 120  $\mu$ M formycin B, or 120  $\mu$ M 5'-deoxyformycin A produced an arrest of cell growth for one day and small increases in the nonviable cell count as determined by the trypan blue exclusion method. Although 5'-deoxyformycin A (gift of Dr. L.B. Townsend) is not converted to a 5'-nucleotide by enzymic phosphorylation, the nucleoside is a cytotoxic agent. The cell counts increased during an additional two-day "recovery" incubation in growth medium. Addition of 50  $\mu$ M dilazep two hr after toxin treatment produced an accelerated two-day recovery of cell growth in comparison to controls. (Dilazep is a vasodilator and an inhibitor of

nucleoside transport.) There is little information available on the mechanism of cell recovery from the action of different toxicants. These findings indicate an intracellular-extracellular turnover of nucleoside toxins. The cell recovery technique could be useful in drug selectivity studies of transformed cells. (See section 6-f for S.O.T. Meeting, February, 1987.)

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TABLE 1  
EFFECT OF PERFLUORO-n-DECANOIC ACID ON EFFLUX OF 2-AMINOPURINE  
FROM L5178Y CELLS<sup>a</sup>

| Treatment                    | AP Efflux<br>Percentage<br>of Controls <sup>b</sup> | Cell Count<br>(cells x 10 <sup>-5</sup> /ml) | Nonviable Count<br>(cells x 10 <sup>-5</sup> /ml) |
|------------------------------|---|--|---|
| Control                      | 100.0 ± 5.3   | 20.5 ± 0.6                                   | 1.0 ± 0.1   |
| PFDA, 100 µg/ml              | 31.5 ± 5.9  | 20.5 ± 0.5                                   | 1.3 ± 0.1   |
| PFDA, 150 µg/ml              | 19.5 ± 7.6  | 19.1 ± 0.6                                   | 1.2 ± 0.2   |
| PFDA, 200 µg/ml <sup>c</sup> | 2.7 ± 0.2   | 16.6 ± 0.8                                   | 1.3 ± 0.1   |
| PFOA, 200 µg/ml <sup>d</sup> | 91.5 ± 3.5  | 19.6 ± 0.6                                   | 0.9 ± 0.1   |
| Ethanol, 300 µg/ml           | 98.1 ± 5.9  | 19.2 ± 0.2                                   | 1.0 ± 0.2   |

<sup>a</sup>Treatment of  $19.0 \times 10^5$  cells/ml for 24 hr at 30°C and AP efflux at 21°C;  
 $\bar{x} \pm SD$ , N = 2.

<sup>b</sup>Rate of AP efflux for  $10^{12}$  cells/liter in comparison with controls.

<sup>c</sup>Cell clumping observed.

<sup>c,d</sup>Dissolved in 300 µg/ml ethanol in growth medium. PFOA is pentadecafluoro-octanoic acid.

TABLE 2

2-AMINOPURINE DETECTED IN PRELOADED L5178Y CELLS<sup>a</sup>

| Treatment                 | AP Content<br>( $\mu\text{M}$ in $10^{12}$ cells/liter) | Cell Count<br>(cells $\times 10^{-6}$ /ml) |
|---------------------------|---|--|
| Control                   | $57.8 \pm 7.1$  | $13.2 \pm 2.0$                             |
| PFDA-treated <sup>b</sup> | $52.4 \pm 3.9$  | $9.6 \pm 0.7$                              |

<sup>a</sup> $\bar{x} \pm \text{SD}$ ,  $N = 2$ .

<sup>b</sup>Cells were incubated in 150  $\mu\text{g}/\text{ml}$  PFDA in McCoy's medium for 24 hr at 30°C. Excess PFDA was removed and the cells were preloaded with 100  $\mu\text{M}$  AP. The cells were rinsed with cold PBS and homogenized; the homogenate was centrifuged and the AP in the supernatant was determined from the emission at 370 nm.

TABLE 3  
 INACTIVATION AND RECOVERY OF 2-AMINOPURINE EFFLUX BY PFDA-TREATMENT  
 AND INCUBATION IN FRESH GROWTH MEDIUM AT 30°C<sup>a</sup>

| Time<br>(days) | AP Efflux <sup>b</sup><br>Percentage<br>of Controls | PFDA-treated <sup>c</sup><br>Percentage Increase<br>in Cell Count | Controls <sup>d</sup><br>Percentage Increase<br>in Cell Count |
|----------------|---|---|---|
| 1              | 20.9 ± 6.9  | 13.5 ± 0.5  | 15.5 ± 0.9  |
| 2              | 21.7 ± 2.1  | 52.2 ± 1.1  | 35.4 ± 3.1  |
| 3              | 22.7 ± 2.0  | 63.0 ± 3.8  | 54.2 ± 3.6  |
| 6              | 65.1 ± 13.9   | 179.0 ± 6.5   | 72.9 ± 4.2  |
| 7              | 70.7 ± 9.5  | 179.0 ± 7.6   | 83.3 ± 7.3  |

<sup>a</sup>L5178Y cells ( $1.7 \times 10^6$  cells/ml) were treated with 150 µg/ml PFDA for 24 hr at 30°C. The cells were rinsed and incubated in fresh medium for 7 days at 30°C.

<sup>b</sup>Rate of AP efflux at 21°C for  $10^{12}$  cells/liter in comparison with controls.  
 $\bar{x} \pm \text{SD}$ , N = 3.

<sup>c</sup>Cell counts in comparison to the count at the start of recovery,  $1.8 \times 10^6$  cells/ml.  $\bar{x} \pm \text{SD}$ , N = 2.

<sup>d</sup>The initial cell count was  $2.1 \times 10^6$  cells/ml.

TABLE 4  
RECOVERY OF 2-AMINOPURINE EFFLUX BY INCUBATION IN FRESH GROWTH MEDIUM<sup>a</sup>

| Treatment      | AP Efflux <sup>b</sup><br>Percentage<br>of Controls | PFDA-Treated <sup>c</sup><br>Percentage Increase<br>in Cell Count | Controls <sup>c</sup><br>Percentage Increase<br>in Cell Count |
|----------------|---|---|---|
| 1 day at 37°C  | 37.1 ± 0.3  | 28.6  | 44.0  |
| 2 days at 37°C | 64.1 ± 1.9  | 105.0   | 122.0   |
| 4 days at 37°C | 72.7 ± 4.0  | 309.0   | 225.0   |
| 9 days at 21°C | 63.1 ± 3.1  | 7.9   | 15.2  |

<sup>a</sup>L5178Y cells were treated with 150 µg/ml PFDA for 24 hr at 30°C. The cells were rinsed and incubated again in fresh medium (the time and temperatures are indicated above).

<sup>b</sup>Rate of AP efflux at 21°C for  $10^{12}$  cells/liter in comparison with controls  $\bar{x} \pm SD$ , N = 2.

<sup>c</sup>Cell counts in comparison to count at the start of recovery,  $1.1 \times 10^6$  cells/ml, N = 2.

TABLE 5  
RECOVERY OF CELL GROWTH BY INCUBATION IN MEDIUM AND DILAZEP<sup>a</sup>

| Treatment                            | Dilazep    | Nonviable <sup>b</sup><br>Count<br>(Percentage) | Cell Count<br>Day 1<br>(Cells x 10 <sup>-5</sup><br>per ml) | Relative Cell <sup>c</sup><br>Count |       |
|--------------------------------------|------------|---|---|-------------------------------------|-------|
|                                      |            |   |   | Day 2                               | Day 3 |
| None                                 | None       | 1.5   | 5.3   | 2.60                                | 4.20  |
| None                                 | 50 $\mu$ M | 1.5   | 4.3   | 2.40                                | 3.70  |
| Formycin A,<br>5.0 $\mu$ M           | None       | 13.0  | 1.5   | 1.60                                | 2.10  |
| Formycin A,<br>5.0 $\mu$ M           | 50 $\mu$ M | 7.0   | 2.0   | 2.20                                | 5.80  |
| Formycin B,<br>120 $\mu$ M           | None       | 6.0   | 3.3   | 2.00                                | 4.10  |
| Formycin B,<br>120 $\mu$ M           | 50 $\mu$ M | 4.5   | 3.4   | 2.10                                | 4.70  |
| 5'-Deoxyfor-<br>mycin A, 120 $\mu$ M | None       | 0   | 4.5   | 2.20                                | 2.50  |
| 5'-Deoxyfor-<br>mycin A, 120 $\mu$ M | 50 $\mu$ M | 3.5   | 3.7   | 2.40                                | 3.60  |

<sup>a</sup>L5178Y cells ( $2.0 \times 10^5$  cell/ml) were incubated with a formycin derivative in growth medium for 2.0 hr at 37°C. Dilazep was added to a final concentration of 50  $\mu$ M and the cells were incubated for an additional 3 days. Cells were counted at the end of each day.  $\bar{x}$ , N = 2.

<sup>b</sup>Nonviable count was determined by the trypan blue exclusion method at the end of day 1.  $\bar{x}$ , N = 2.

<sup>c</sup>Cell counts after these intervals in comparison to the count at the end of day 1.



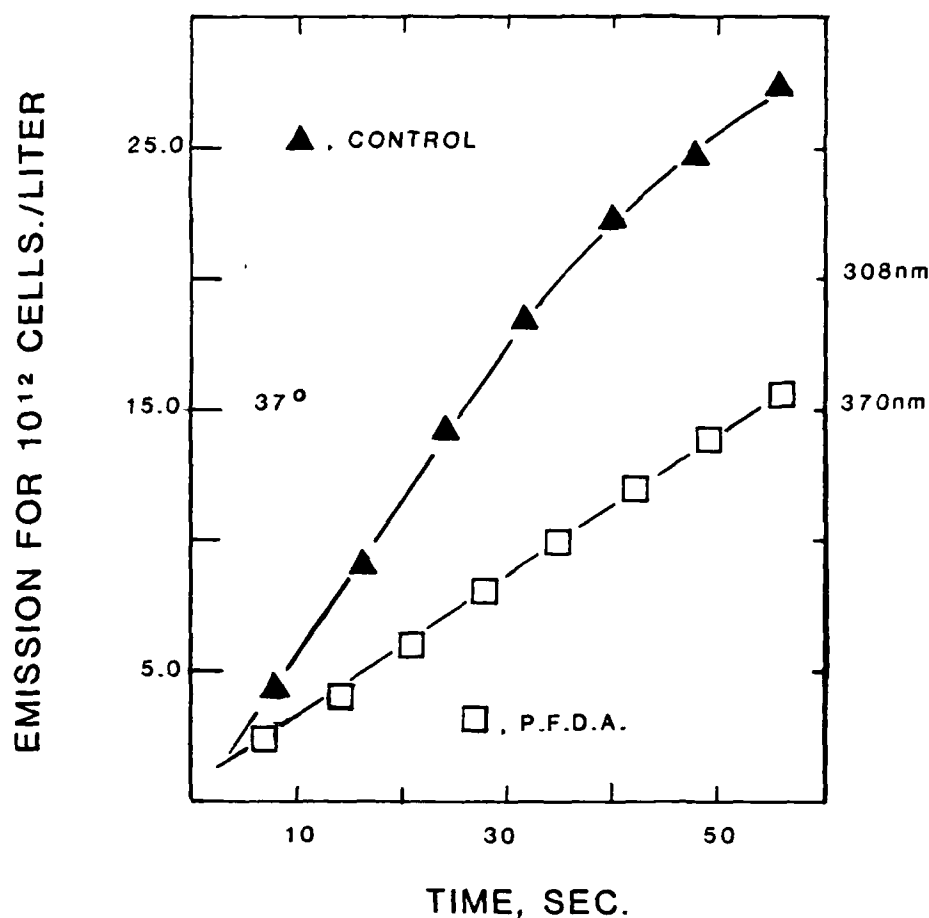


Fig. 1. Effect of a Prior Incubation of L5178Y Cells with 100  $\mu\text{g/ml}$  PFDA in Medium for 24 hr at 37° on the Efflux of AP. Cells were incubated with PFDA (□) and no addition (▲). The cells were preloaded with 100  $\mu\text{M}$  AP and the efflux rate was determined with the filtration-flow apparatus at 21°. Excitation was at 308 nm and emission was at 370 nm.

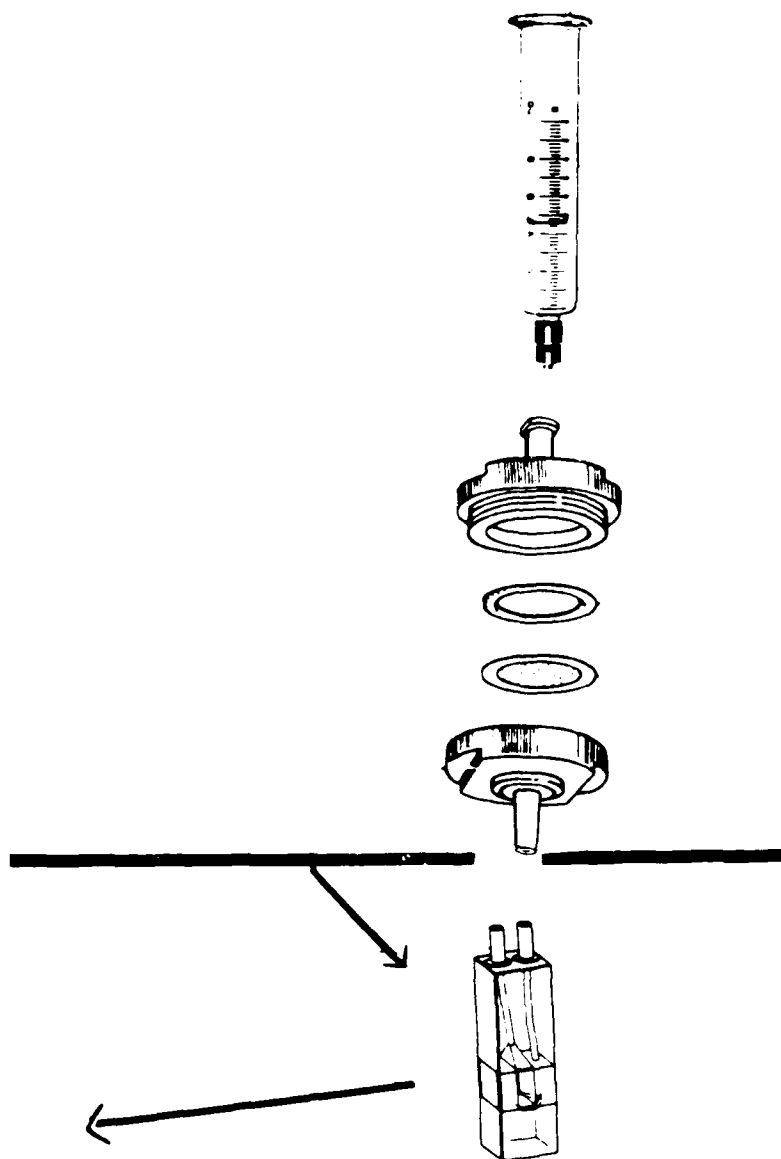


Fig. 2. A sketch of the flow-filtration apparatus for the rapid determination of the rate of 2-aminopurine efflux. The suspension of preloaded cells is placed in a syringe barrel connected to a holder for a 5.0 micron membrane filter. The efflux buffer is drained through a quartz flow cell with medical plastic tubing. The excitation light enters the cell from the rear and the emission light is detected at a right angle.

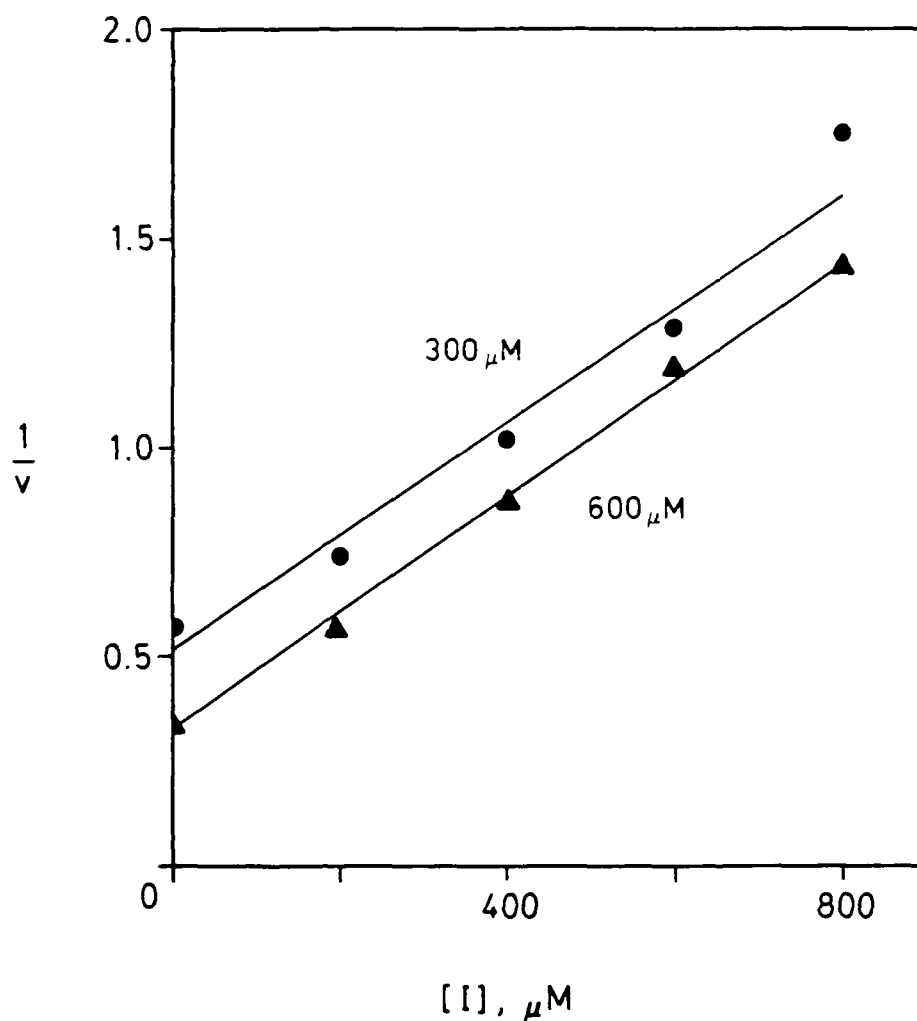


Fig. 3. A plot of  $1/v$  against  $[I]$  that shows the trans inhibition by I (uric acid) of the efflux of AP from the L5178Y cells at pH 7.4 and 21°C. The circles are average experimental values of the reciprocal of the rate of AP efflux and  $[AP] = 300 \mu\text{M}$ ; the triangles represent the corresponding values at  $[AP] = 600 \mu\text{M}$ . The straight lines were plotted from an analysis of least squares.

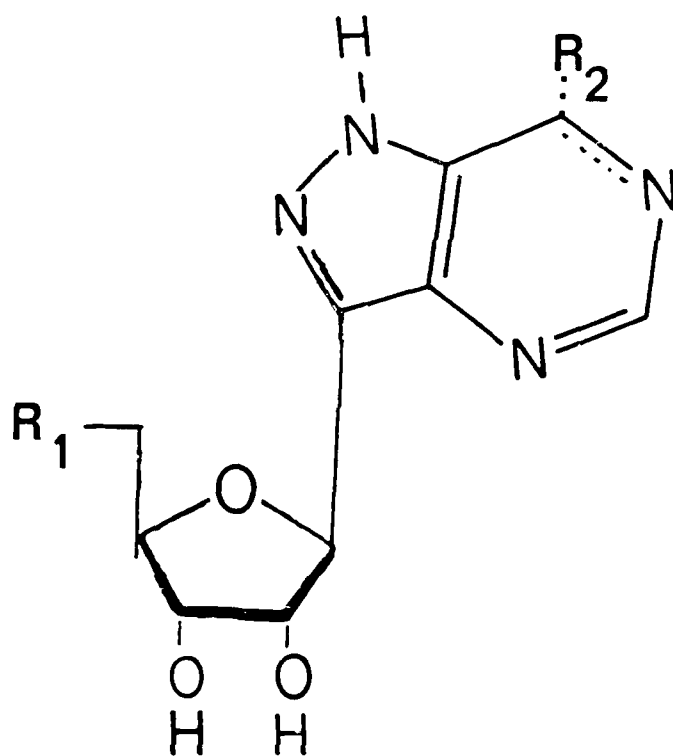


Fig. 4. Formycin A;  
Formycin B;  
5'-deoxyformycin A;

$R_1$  is HO- and  $R_2$  is  $-NH_2$   
 $R_1$  is HO- and  $R_2$  is -O  
 $R_1$  is H- and  $R_2$  is  $-NH_2$

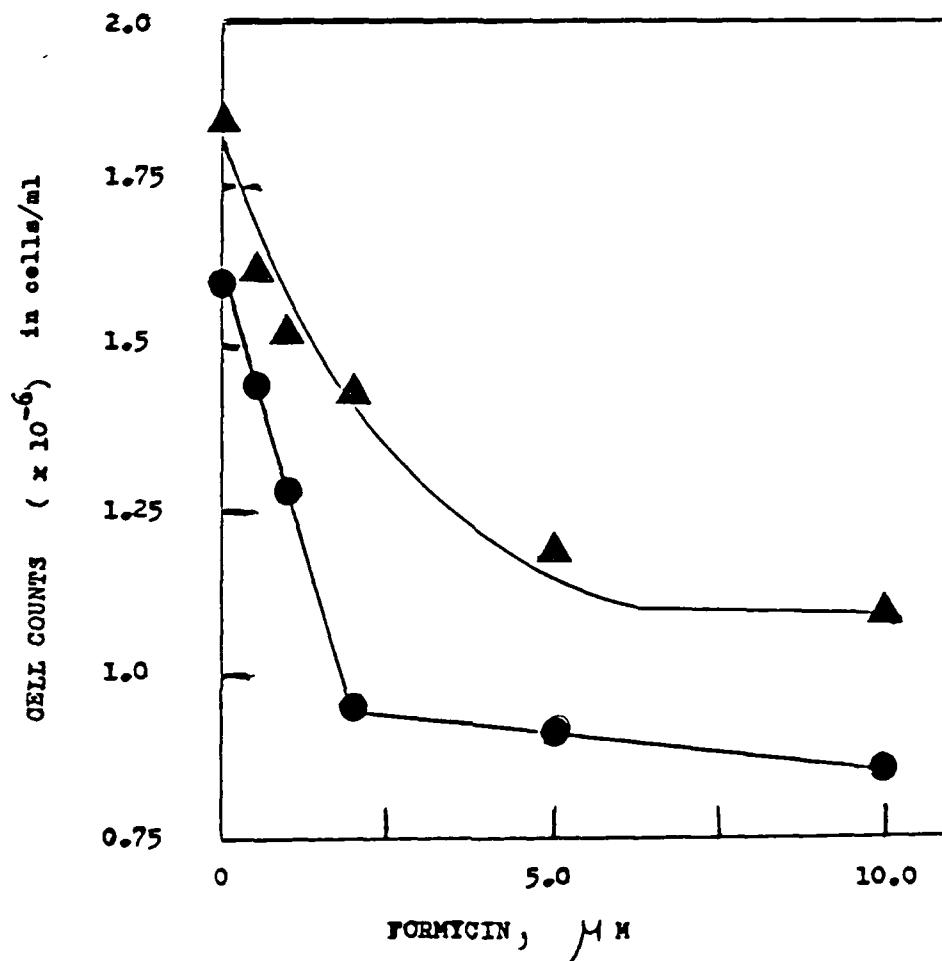


Fig. 5. The Effect of Formycin A at Different Concentrations on the Count of L5178Y Cells. The circles represent the average counts ( $N = 2$ ) determined after 24 hr incubation at 37°C, and the triangles represent the average counts after 48 hr incubation with formycin A in the growth medium. The cell count at time zero was  $1.0 \times 10^6$  cells/ml.

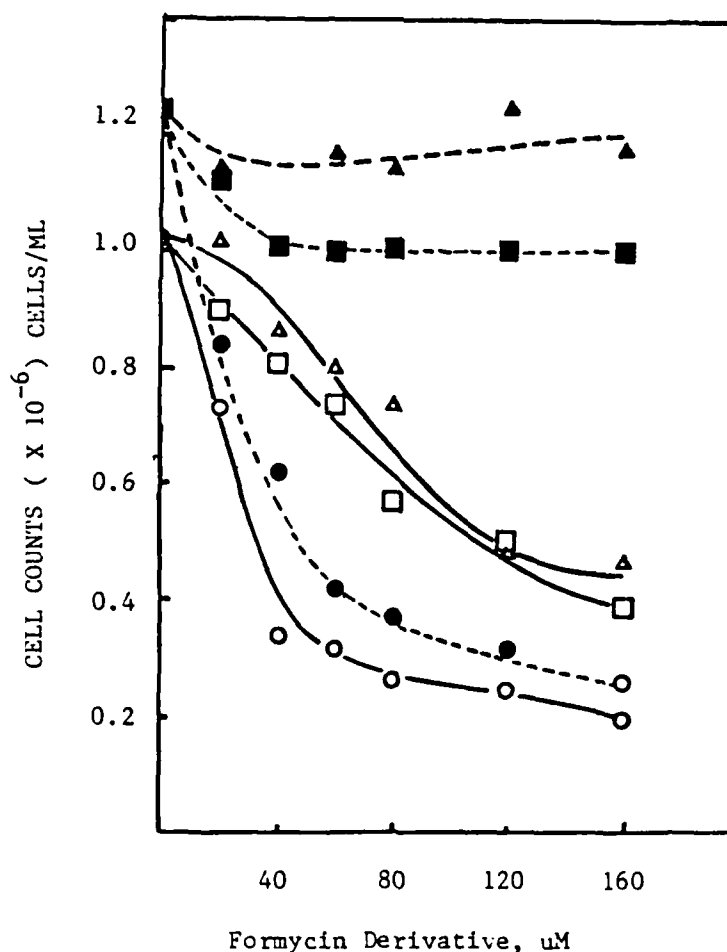


Fig. 6. Effect of Formycin Derivatives at 37°C on Cell Counts. L5178Y cells ( $0.5 \times 10^6$  cells/ml) in 5 ml growth medium were incubated with formycin derivatives at different concentrations for two days. The cells were counted at the end of each day. ○—○, formycin A day 1; ●--●, formycin A day 2; □, formycin B day 1; ■, formycin B day 2; △, 5'-deoxyformycin A day 1; ▲, 5'-deoxyformycin A day 2.

4) Publications:

- a) P. Wigler, Annual Report for AFOSR Grant 82-0261A. Submitted on October 11, 1983. "Effect of Chemicals on the Cell Membrane Transport of Nucleosides."
- b) P. Wigler, Annual Report for AFOSR Grant 82-0261B-C. Submitted on September 15, 1984. "Effect of Perfluorodecanoic Acid on the Efflux of 2-Aminopurine from L5178Y Cells."
- c) P. Wigler, Annual Report for AFOSR Grant 82-0261D-E. Submitted on July 15, 1985. "Perfluorodecanoic Acid Inactivation of a Channel for 2-Aminopurine in the L5178Y Cell Membrane and Recovery of the Purine Channel."
- d) P.W. Wigler (1986) "5- $\beta$ -D-Ribofuranosyl-4-thiouracil (4-thiopseudouridine) a nonmetabolized transport substrate of known structure." In: Nucleic Acid Chemistry: Improved and New Synthetic Methods and Techniques (L.B. Townsend and R.S. Tipson, eds.). John Wiley and Sons, Inc., New York, pp. 91-94.
- e) P.W. Wigler and Y.B. Shah (1986) "Perfluorodecanoic acid inactivation of a channel for 2-aminopurine in the L5178Y cells membrane and recovery of the channel." Toxicology and Applied Pharmacology (in press).
- f) P.W. Wigler and Y.B. Shah (1987) "Effects of formycins and dilazep on growth arrest and recovery of L5178Y cells." Toxicology and Applied Pharmacology (in preparation).

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6) Papers Presented at Meetings and Conferences:

- a) P.W. Wigler and Y.B. Shah, July 26, 1983, "Effects of Perfluorodecanoic Acid on Transport in L5178Y Cells." Review of Air Force Sponsored Basic Research in Biomedical Sciences, University of California, Irvine, Calif. Paper No. 10.
- b) P.W. Wigler and Y.B. Shah, November 11, 1983, invited symposium paper, "Toxicant Effects on the Efflux Rate of Fluorescent Substrates from

L5178Y Cells." American Chemical Society 35th Southeast Regional Meeting, Charlotte, NC. Paper No. 230, Symposium Title "Structure and Transport in Membranes."

- c) P.W. Wigler and Y.B. Shah, December 10, 1983, "Effects of Perfluorodecanoic Acid on a Purine Transporter." Southeastern Regional Chapter Meeting of the Society of Toxicology, Oak Ridge Associated Universities, Oak Ridge, Tennessee.
- d) P.W. Wigler and Y.B. Shah, October 29, 1984. "Recovery of a purine transporter from the action of a toxicant, perfluorodecanoic acid." Review of Air Force Sponsored Basic Research on Membrane Bioeffects, Wright-Patterson AFB, Dayton, Ohio. Paper No. 3.
- e) P.W. Wigler and Y.B. Shah, November 29, 1984. "Recovery of a purine transporter from the action of a toxicant, perfluorodecanoic acid." Fifth annual meeting of the American College of Toxicology, Crystal City, VA. Paper No. 108.
- f) P.W. Wigler and Y.B. Shah, February 23-27, 1987. "Effects of Formycins and Dilazep on Growth Arrest and Recovery of L5178Y Cells." Society of Toxicology 26th Annual Meeting, Washington, D.C. (to be submitted) (abstract enclosed).

Visiting Lectures:

- g. Seminar to staff of U.S. Army Medical Research Institute of Chemical Defense, Aberdeen P.G., Maryland, April 18, 1984. "Effect of Perfluorodecanoic Acid on Efflux of 2-Aminopurine from L5178Y Cells."
- h. Seminar to members of the Life Sciences Directorate, AFOSR, Bolling AFB, Washington, CD, April 20, 1984. "Toxicant Effects on Transport of a Fluorescent Purine in L5178Y Cells."

- 7) Inventions or Patents: NONE



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